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Staphylococcus aureus SarA is a regulatory protein responsive to redox and pH that can support bacteriophage lambda integrase-mediated excision/recombination

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Summary

Staphylococcus aureus **produces a wide array of virulence factors and causes a correspondingly diverse array of infections. Production of these virulence factors is under the control of a complex network of global regulatory elements, one of which is *sarA*. *sarA* encodes a DNA binding protein that is considered to function as a transcription factor capable of acting as either a repressor or an activator. Using competitive ELISA assays, we demonstrate that SarA is present at approximately 50 000 copies per cell, which is not characteristic of classical transcription factors. We also demonstrate that SarA is present at all stages of growth *in vitro* and is capable of binding DNA with high affinity but that its binding affinity and pattern of shifted complexes in electrophoretic mobility shift assays is responsive to the redox state. We also show that SarA binds to the bacteriophage lambda (λ) attachment site, *attL*, producing SarA-DNA complexes similar to intasomes, which consist of bacteriophage lambda integrase, *Escherichia coli* integration host factor and *attL* DNA. In addition, SarA stimulates intramolecular excision recombination in the absence of λ excisionase, a DNA binding accessory protein. Taken together, these data suggest that SarA may function as an architectural accessory protein.**

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Introduction

Staphylococcus aureus is a major health concern based largely on the continued emergence of antibiotic-resistant strains. In fact, a recent report concluded that methicillin-resistant *S. aureus* caused approximately 94 360 invasive infections in 2005, with 18 650 of these resulting in death (Klevens *et al.*, 2007). This means that *S. aureus* has passed AIDS as a cause of death in the United States. This same study also documented the continued emergence of community-acquired methicillin-resistant *S. aureus* isolates capable of causing serious infections even in the absence of recognized risk factors. Among the most devastating of these infections is necrotizing pneumonia (Bradley, 2005), but *S. aureus* also causes a diverse array of other infections including osteomyelitits, septic arthritis and endocarditis (Lowy, 1998). Although not strictly an infection, *S. aureus* is also estimated to cause more than 185 000 cases of food poisoning each year in the United States alone (Mead *et al.*, 1999), and it was estimated to be responsible for 16% of all food-borne disease in France from 1999 to 2000 (Le Loir *et al.*, 2003). *S. aureus* also has a large impact on the food industry, with staphylococcal bovine mastitis costing the US dairy industry billions of dollars annually (Donovan *et al.*, 2005).

The capacity of *S. aureus* to cause such a diverse array of infections is due to its ability to produce a diverse array of virulence factors in a controlled fashion in response to changing environmental conditions within the host. These virulence factors can be broadly divided into three groups. The first are surface-exposed proteins and polysaccharides that play an important role in immune avoidance and colonization of host tissues (Foster, 2005). The second are extracellular virulence factors that include both degradative enzymes and a variety of toxins, some of which are directly responsible for the observed pathology of the corresponding infection (Dinges *et al.*, 2000; Murray, 2005). The third are the regulatory elements that modulate the production of different virulence factors, often on

a global scale (Novick, 2003; Bronner *et al*., 2004; George and Muir, 2007; Cheung *et al*., 2008). Although there are exceptions, the general regulatory paradigm is that surface proteins are produced during the exponential growth phase while the extracellular enzymes and toxins are produced after the transition into post-exponential growth (Novick, 2003). While based primarily on *in vitro* experiments, this paradigm is thought to have an *in vivo* corollary that corresponds to the early versus late stages of infection (Cheung *et al*., 2008).

A number of regulatory loci interact in complex ways to modulate this transition, but it is generally accepted that the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sarA*) play central roles in that regard (Novick, 2003; Bronner *et al*., 2004; Cheung *et al*., 2004; George and Muir, 2007). The *agr* regulatory system consists of the *agr* operon itself (*agrBDCA*), which encodes the components of a two-component, quorum-sensing system, and a divergently transcribed region encoding a regulatory RNA designated RNAIII (Novick, 2003; Lyon and Novick, 2004). RNAIII production characteristically occurs during the transition to post-exponential growth and results in increased production of most extracellular virulence factors and decreased production of most surface-associated proteins. Genome-scale transcriptional profiling experiments have confirmed that mutation of *agr* results in global changes in gene expression (Dunman *et al*., 2001; Cassat *et al*., 2006). Although the impact is somewhat strain-dependent (Blevins *et al*., 2002), mutation of *agr* has been associated with reduced virulence in every strain and every animal model studied to date (Abdelnour *et al*., 1993; Cheung *et al*., 1994; Booth *et al*., 1995; Gillaspy *et al*., 1995).

The *sarA* locus was originally identified in the *S. aureus* strain DB, and based on phenotypic characterization it was described as an 'anti-*agr*' regulatory element (Cheung *et al*., 1992). However, when examined in the more commonly studied genetic background of 8325-4 strains, *sarA* was found to enhance rather than antagonize *agr* function (Chien *et al*., 1998). To date, the only recognized functional product of the *sarA* locus is a single polypeptide (SarA) that dimerizes to form a winged helix DNA binding protein (Morfeldt *et al*., 1996; Chien and Cheung, 1998; Rechtin *et al*., 1999; Cheung *et al*., 2008). SarA modulates gene expression by binding to DNA elements *cis* to its target genes (Cheung and Projan, 1994; Chien and Cheung, 1998; Blevins *et al*., 1999; Rechtin *et al*., 1999; Sterba *et al*., 2003), and it has been assumed on this basis that SarA functions as a classic transcription factor capable of activating expression of certain genes (e.g. *agr*, *hla*) and repressing expression of others (e.g. *cna, sspA*). Mutation of *sarA* was also recently shown to result in de-stabilization of mRNA transcripts (Roberts *et al*., 2006), although at present it is unclear whether this is a direct or indirect effect of SarA binding.

A number of important questions remain regarding how SarA modulates gene expression. For example, *sarA*-mediated regulation clearly has a temporal component, which is reflected at least to some degree in the temporal pattern of transcription from each of the *sarA* promoters (Manna *et al*., 1998), but to date there is no consensus with respect to the growth phase-dependent production of functional SarA. Blevins *et al.* (1999) concluded that SarA is present in equivalent amounts throughout the *in vitro* growth cycle, while Manna and Cheung (2001) found that SarA levels are highest in exponential-phase cultures and decline during the post-exponential growth phase. In contrast, Chan and Foster (1998) found that SarA activity is highest in post-exponential cultures. These latter authors also found that SarA activity is enhanced under microaerophilic conditions.

A second unresolved issue is the lack of a clear definition for a functional DNA binding site. This reflects the fact that SarA has the capacity of binding DNA at relatively diverse sites, the only clear consensus being its preference for AT-rich DNA (Chien *et al*., 1999; Sterba *et al*., 2003; Gao and Stewart, 2004; Cheung *et al*., 2008). Such sites occur repeatedly throughout the AT-rich *S. aureus* genome. Partly for this reason, it has been suggested that SarA may function as an architectural protein that recognizes and binds distinct topological features in the target DNA (Fujimoto *et al*., 2000). Support for this hypothesis comes from the observation that the –10 and –35 regions in the RNAIII promoter, expression of which is under the regulatory control of SarA, are spaced further apart than normal and, more importantly, that deletion of 3 bp between these elements not only restores the correct spacing, but also diminishes the impact of SarA on *agr* transcription (Morfeldt *et al*., 1996).

Several *Escherichia coli* proteins have been identified that modulate gene expression and function, at least in part, by altering DNA topology. These include integration host factor (IHF) and heat unstable (HU) protein (Johnson *et al*., 2005). IHF was originally identified as a bacterial factor that contributes to Int-mediated site-specific recombination in bacteriophage λ (Nash, 1990). More specifically, Int is involved in both integration and excision of the bacteriophage λ genome into and out of the *E. coli* chromosome. Integration requires specific sites within the bacteriophage and bacterial genomes (*attP* and *attB* respectively) and results in formation of hybrid *att* sites designated *attL* and *attR* (Azaro and Landy, 2002). When bacteriophage λ exits the *E. coli* chromosome, *attL* and *attR* are brought together forming a structurally distinct excision protein–DNA complex with the DNA binding accessory proteins, IHF, excisionase (Xis), a recombination directionality factor and Int

(Abremski and Gottesman, 1982; Moitoso de Vargas and Landy, 1991).

In this report, we sought to investigate whether SarA may operate in *S. aureus* in a manner similar to that observed with architectural proteins in *E. coli*. Specifically, we demonstrate that SarA is present at intracellular concentrations far exceeding any classical transcription factor and that its concentration remains essentially unchanged during different phases of *in vitro* growth. However, we also found that SarA-dependent DNA binding activity was highest in the exponential growth phase and that the decline is SarA activity during the post-exponential growth phase is associated with changes in both redox potential and pH. Finally, we demonstrate that SarA can bind the bacteriophage λ hybrid attachment site *attL* and that it can at least partially replace the Xis architectural DNA binding protein involved in Int-mediated intramolecular excision.

Results

Accumulation and concentration of SarA in S. aureus *cells*

Using a polyclonal anti-SarA antibody (Blevins *et al.*, 1999), we performed Western blot analysis with cell lysates prepared from RN6390 and its isogenic *sarA* mutant UAMS-957. Lysates were prepared using two methods (see *Experimental procedures*) from cells harvested from tryptic soy broth cultures at the early exponential (A_{595} = 0.3), late exponential (A_{595} = 1.0) and stationary growth phases (A_{595} = 4.0). Irrespective of the lysis method employed, there were similar levels of SarA present at all growth phases in RN6390 (Fig. 1). Western blots done with lysates from the UAMS-957 *sarA* mutant confirmed the specificity of this assay. These results are consistent with our previous observations focusing on both RN6390 and the clinical isolate UAMS-1 (Blevins *et al.*, 1999).

Using competitive ELISAs increasing amounts of purified SarA were shown to limit the amount of SarA available for binding in the ELISA assay as evidenced by reduced HRP activity, with ~40 ng of purified SarA being required to bind 50% of the available antibody (Fig. 2A). Competitive inhibition was also observed with cell-free lysates, with 50% inhibition being observed with ~25 µl of lysate (Fig. 2B). The binding curves were very similar with lysates prepared from all growth phases (Fig. 2B). Based on the number of cells used to prepare the lysates (see *Experimental procedures*), these results indicate that a single *S. aureus* cell contains ~50 000 dimers of SarA at all stages of growth *in vitro*.

The temporal pattern of SarA DNA binding activity

The observation that the concentration of SarA does not change in a growth phase-dependent manner does not mean that its activity also remains unchanged. To address this issue, extracts from the same growth phases tested above were used in electrophoretic mobility shift assays (EMSA) using DNA binding targets associated with *agr*, *sspA* and *cna*. The DNA binding activity of SarA was slightly higher in the post-exponential phase than exponential phase and lowest in stationary phase (Fig. 3). However, as the extract dilutions are only twofold, the difference in DNA binding activity between the different growth phases is very small. As with our Western blot analysis, concomitant experiments done with the UAMS-957 *sarA* mutant confirmed the specificity of this binding.

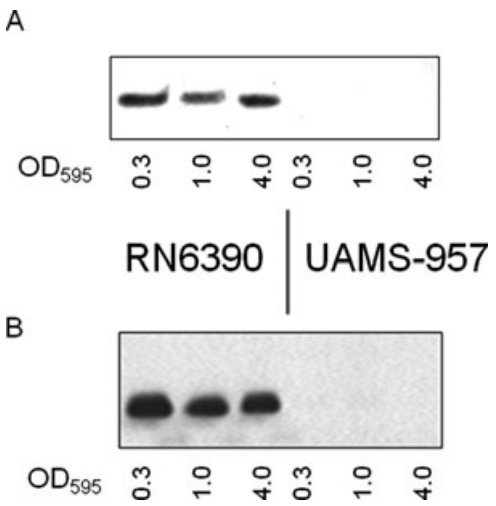


Fig. 1. Western blot analysis of SarA accumulation.
A. Cells were lysed gently with lysostaphin.
B. Cells were lysed by FastPrep method. 0.3, 1.0 and 4.0 indicate the optical density of cells in pre-log phase, post-log phase and stationary phase respectively. SarA was detected with polyclonal anti-SarA IgG as described in *Experimental procedures*. RN6390 is the wild-type strain and UAMS-957 is its *sar*⁻ mutant.

SarA DNA binding activity is responsive to redox conditions

It was previously reported that SarA activity is enhanced in laboratory cultures grown under microaerobic conditions (Chan and Foster, 1998; Lindsay and Foster, 1999). This raises the possibility that SarA function is responsive to oxygen tension. To test this possibility, we examined the DNA binding activity of SarA under various redox conditions. The results confirmed that the binding affinity of SarA for the *agr* B1/B2 binding site increases with the level of reducing agent and decreases with the level of oxidizing agent (Fig. 4A). SarA has a single cysteine at position 9. To test the importance of this cysteine in the redox response we generated a C9A *sarA* mutant and purified the variant protein to apparent homogeneity (data not shown). EMSA analysis confirmed that replacement of the cysteine at position 9 eliminated the redox responsive

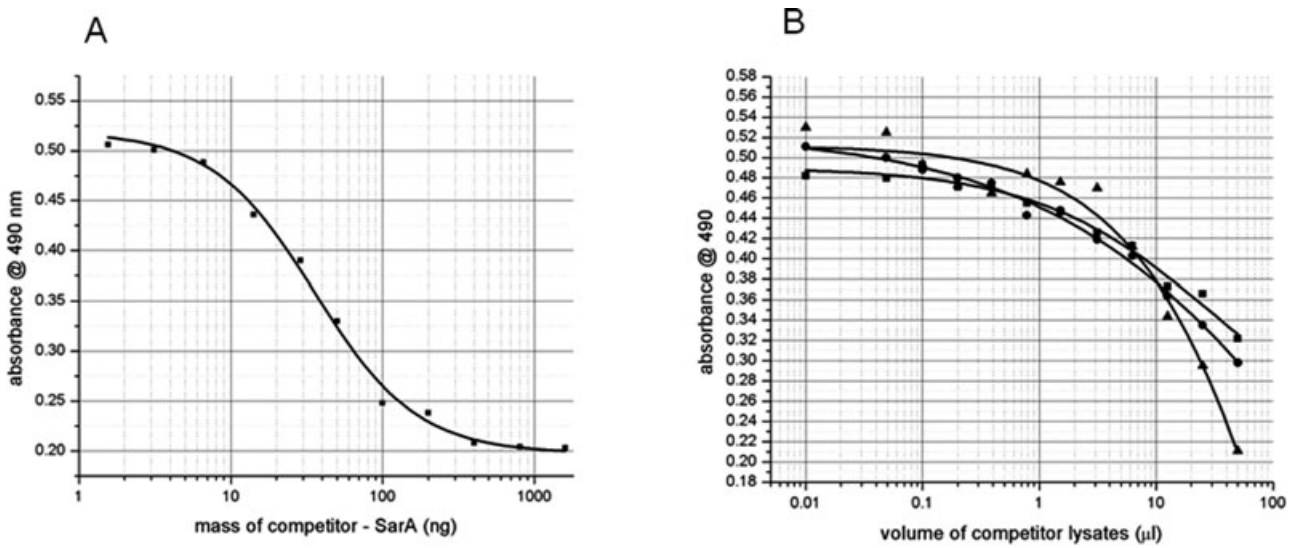


Fig. 2. Competitive ELISA analysis of SarA accumulation.
A. Purified recombinant SarA was used to generate a standard curve for inhibition of IgG binding to SarA adsorbed to the microtiter wells. The mass of SarA is plotted versus the absorbance of the chromophore used to detect IgG bound to the plate.
B. Crude lysates of UAMS-179 (*spa*⁻) from pre-log (■), post-log (●) and stationary (▲) phases are plotted versus absorbance of the chromophore used to detect IgG bound to the plate.

nature of SarA binding and resulted in a DNA binding affinity comparable to that observed with the native protein under non-reducing conditions (Fig. 4B).

SarA DNA binding activity is responsive to pH

In addition to redox, pH is another environmental factor that changes depending on growth conditions. Indeed, expression of *agr* is sensitive to pH changes (Regassa and Betley, 1992; Regassa *et al.*, 1992). In addition, Weinrick *et al.* (2004) recently demonstrated that exposure to mildly acidic conditions results in global changes in *S. aureus* gene expression. Although it was unclear whether this was a direct or indirect effect, *sarA* was shown to be involved in some fashion in the adaptive response to low pH. To more directly examine whether SarA activity is pH-dependent, we also determined the relative binding affinity of SarA and the C9A SarA variant under varying pH conditions. The results demonstrate that the DNA binding affinity of SarA is highest at low pH (Fig. 5A) where the most dilute extract still has significant shifted complexes and the higher pH samples required

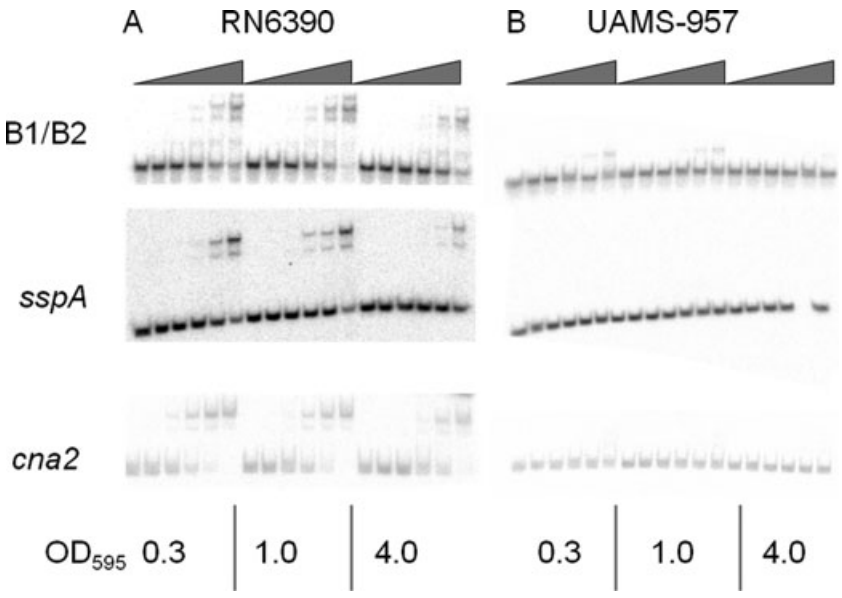


Fig. 3. EMSA analysis of cell-free extracts of RN6390 (A) and UAMS-957 (B). Increasing amounts of extracts were tested with the B1/B2 SarA binding site from the *agr* regulatory region, the SarA binding site from the serine protease gene *sspA* and the SarA binding site from the collagen adhesin gene *cna*. The extracts were made from cultures at early log phase (0.3), late log phase (1.0) and stationary phase (4.0).

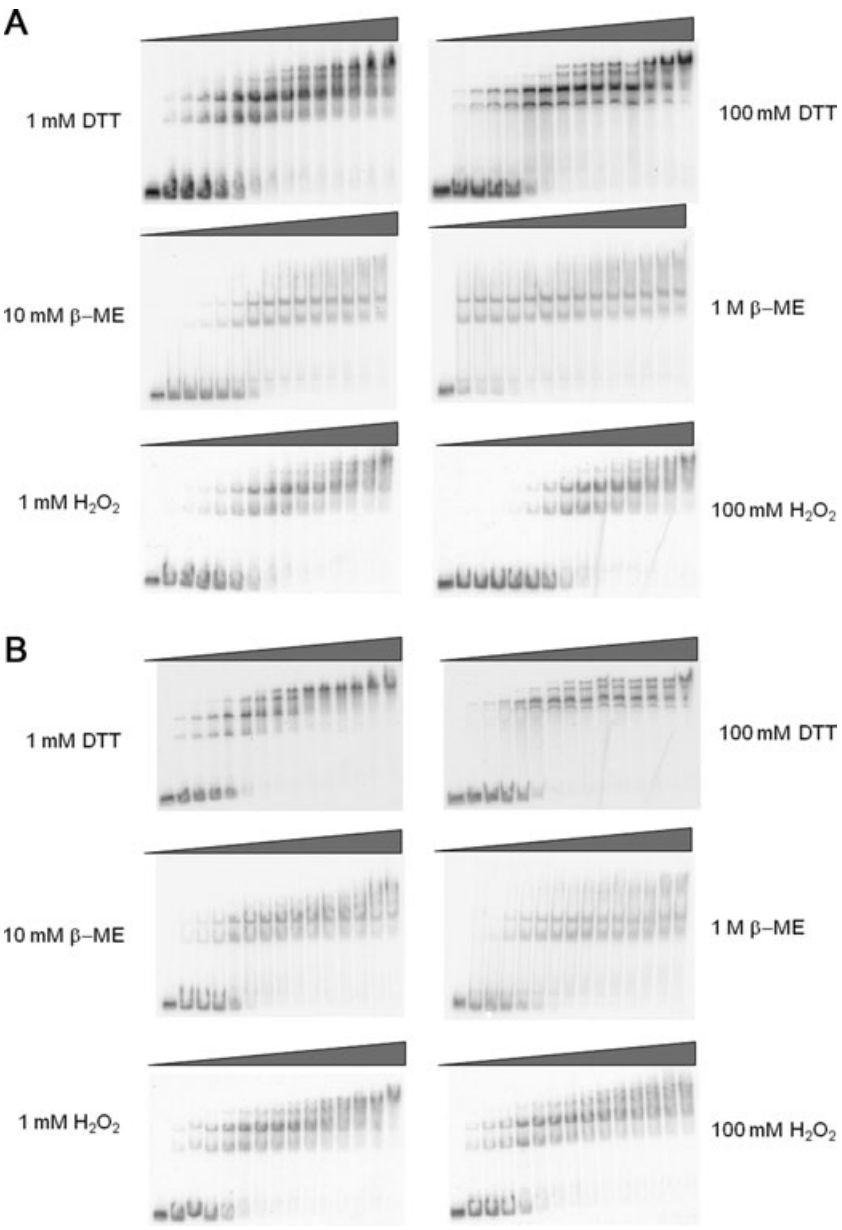


Fig. 4. EMSA analysis of SarA DNA binding under varying redox conditions. The B1/B2 SarA binding site from the *agr* regulatory region was used in all experiments. The redox reagents added to the reaction are indicated. The concentration of SarA increases from right to left as indicated by the triangles. A. Wild-type SarA. B. SarA mutant C9A.

more extract to form complexes. This effect was also at least partially dependent on the cysteine at position 9 (Fig. 5B). It should be noted though, that intracellular pH is not expected to vary to the extent of the *in vitro* experiments reported here. There is not an obvious change in binding between pH 7 and pH 7.5 where intracellular pH is maintained.

*SarA binds to the bacteriophage lambda attachment site (*attL*) and forms an ordered protein DNA complex resembling Int-IHF–*attL *intasomes*

The bacteriophage lambda recombination system has been productive for analysing DNA binding accessory proteins (Nash, 1990; Segall *et al*., 1994). This involves both host (*E. coli*) proteins (e.g. IHF) and proteins encoded by the bacteriophage (e.g. Int). Specifically, IHF bends bacteriophage lambda attachment sites forming distinct complexes with DNA and Int (Nash, 1990; Goodman *et al*., 1992). These higher order nucleoprotein structures are referred to as *attL* intasomes. HU, a second non-specific DNA binding protein in *E. coli* can replace the architectural role of IHF to form an HU-Int–*attL* complex (Segall *et al*., 1994). Relevant protein binding sites in this excision/recombination system are illustrated in Fig. 6A.

To determine whether SarA may serve a similar architectural role, we used a 179 bp DNA fragment containing *attL* (co-ordinates –69 to +110 with respect to the centre of

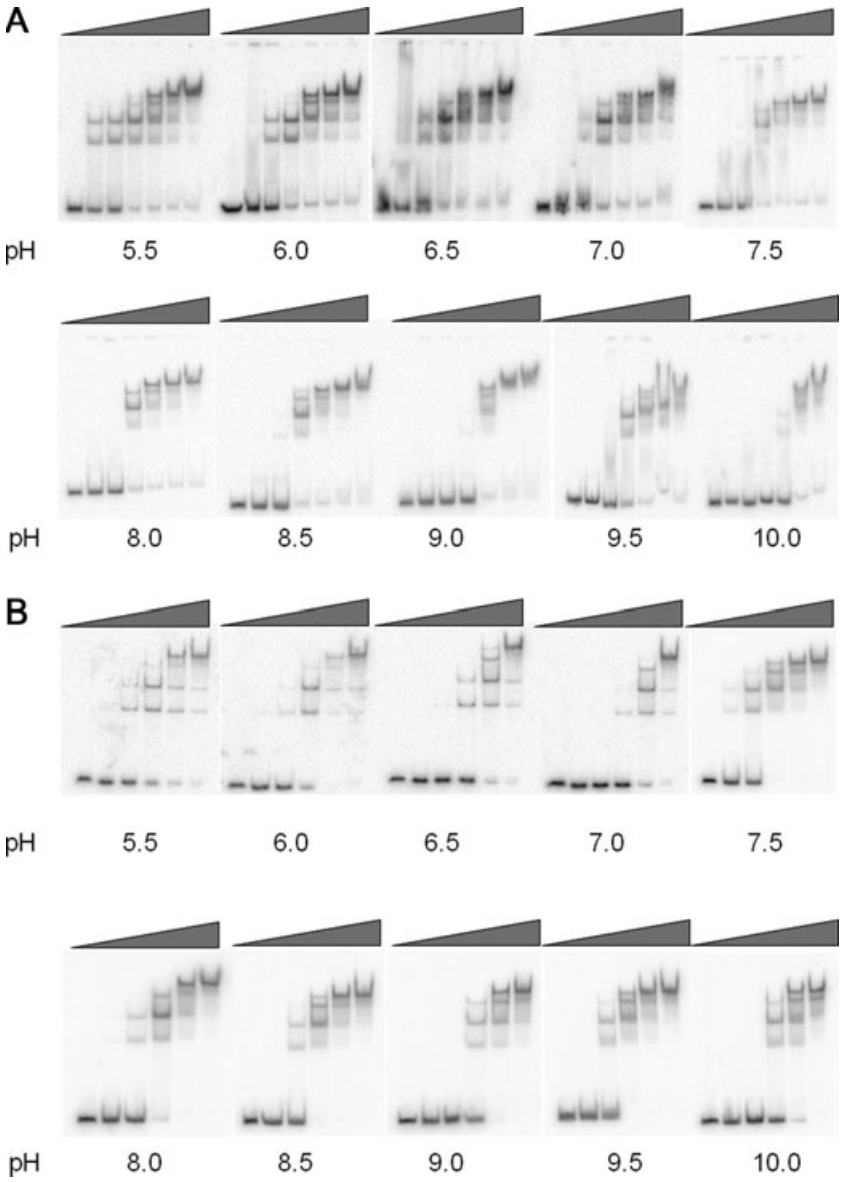
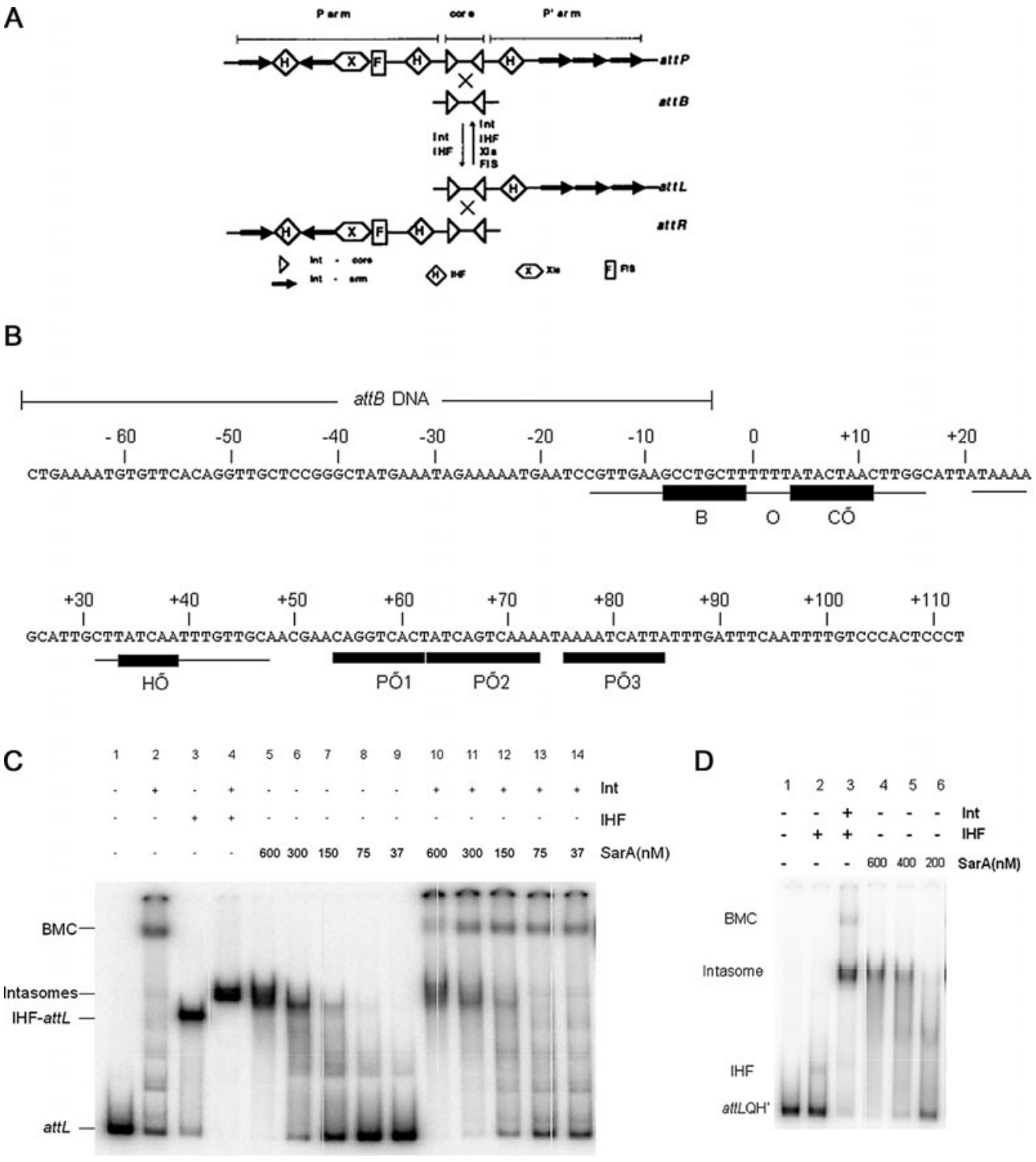


Fig. 5. EMSA analysis of SarA binding DNA under varying pH conditions. The B1/B2 SarA binding site from the *agr* regulatory region was used in all experiments. The pH of the reactions is indicated. The concentration of SarA increases from right to left as indicated by the triangles. A. Wild-type SarA. B. SarA mutant C9A.

Int crossover region, Fig. 6B) and tested whether SarA can replace IHF in complexes with Int. As shown in Fig. 6C, both Int and IHF can bind *attL* (lanes 2 and 3 respectively) and, when included together, can form the distinct intasome nucleoprotein complex (lane 4). When SarA was added alone with *attL*, complexes with a similar migration rate as the Int-IHF–*attL* intasome were observed in a concentration-dependent manner (Fig. 6C, lanes 5–9). This shows that, under conditions that support Int-mediated recombination, SarA bound to *attL* can form

Fig. 6. A. Schematic showing DNA substrates, binding sites and proteins necessary for bacteriophage lambda recombination. Bacteriophage lambda *attP* and the bacterial integration site *attB* are shown. Below, are the hybrid attachment sites, *attL* and *attR*. Triangles = core sites, diamonds = IHF binding sites, hexagons = Xis binding sites and rectangles = Fis binding sites.
B. Sequence of the 179 bp *attL* DNA used for EMSA assays. BOC′ depicts the core site where B represents *attB*, O represents the overlap region, which is homologous to *attB* and *attP*, and C′ represents *attP* DNA.
C. EMSA testing SarA binding to 179 bp *attL* DNA. The proteins added to each reaction mixture are shown above each lane (see *Experimental procedures* for concentrations); the various amounts of SarA added to reactions are represented as concentrations (nM) shown above the gel image. Complexes formed are shown on the left side of the gel image. BMC, bimolecular complex (two *attL* DNA fragments and four Int protomers), *attL* intasome (*attL*, one IHF dimer and one or two Int protomers).
D. EMSA testing SarA binding to *attL*QH′. Reaction conditions are identical as described in Fig. 6C except *attL*QH′ DNA was used as a DNA binding target for SarA.



a complex similar to the intasome. When SarA was added to reactions containing Int, complete incorporation of *attL* DNA into an intasome complex was not observed (Fig. 6C, lanes 10–14), although the addition of 300 and 600 nM SarA in the presence of Int and *attL* formed complexes that migrate at a similar rate as the *attL* intasome (Fig. 6C, lanes 10 and 11) and the SarA–*attL* complex (Fig. 6C, lanes 5 and 6). The simplest interpretation of these results is that Int and SarA compete for binding the *attL* target.

As the SarA used in these experiments was expressed and purified from *E. coli*, we were concerned that a small amount of contaminating IHF may be present in the protein preparation. Therefore, we tested if SarA can bind

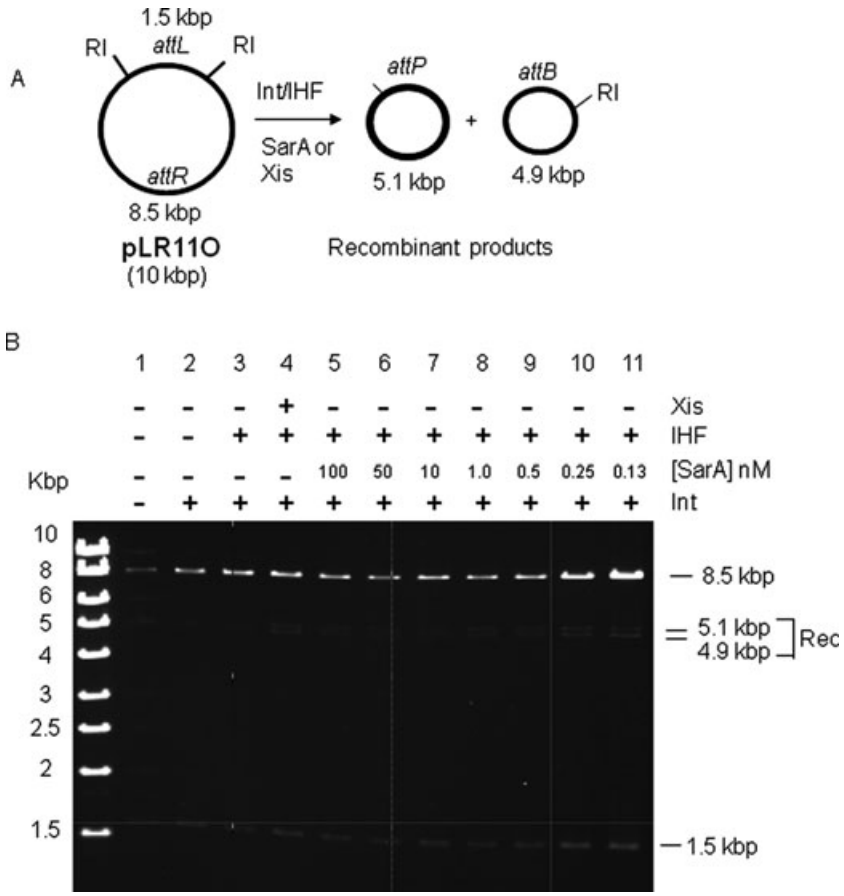


Fig. 7. A. Schematic diagram and physical map showing the excision recombination substrate (pLR110) and products of recombination (Craig and Nash, 1983). RI = EcoRI restriction sites. Sizes of substrate and recombinant products are denoted by size in kbp. The attachment sites, *attL* and *attR* producing *attP* and *attB* are shown on each DNA element.
B. Intramolecular excisive recombination stimulated by SarA in the absence of excisionase. Size of the EcoRI-digested substrate and recombinant products were determined by comparing with the size standard on the left side of the gel image.

to and form a complex with a substrate that has a four-base-pair substitutions that alters the specific IHF binding site (H′) in *attL* to generate a variant called *attL* QH′ (Gardner and Nash, 1986). This mutation decreases the affinity for IHF to less than 1% compared with the wild-type H′ sequence (Fig. 6D, lane 2 compared with Fig. 6C, lane 3). In contrast, IHF was able to form an intasome complex with *attL* QH′ in the presence of Int (Fig. 6C, lane 3). In contrast, SarA and *attL* QH′ formed a complex that migrated similarly to the intasome even in the absence of Int (Fig. 6D, lanes 4 and 5). This demonstrates that the intasome-like complex formed in the presence of SarA purified form *E. coli* is due to SarA binding rather than contaminating IHF.

SarA can replace Xis in recombination/excision

SarA was tested for its ability to replace Xis in the excision recombination reaction using an Int-mediated intramolecular resolution assay with a plasmid containing the prophage attachment sites *attL* and *attR* (Abremski and Gottesman, 1982; Craig and Nash, 1983). A schematic representation of the excision recombination reaction is shown in Fig. 7A. The results confirm that SarA can stimulate excisive recombination in the absence of Xis when IHF and Int are added to the reaction (Fig. 7B). This was true at all the concentrations of SarA tested (Fig. 7B, lanes 5–11). As a positive control, Int, IHF and Xis were added to the same reaction, and comparison of the results demonstrates that 0.5 nM SarA produces similar amounts of recombination products (Fig. 7B, lane 9) by comparison with the Xis-mediated reaction (Fig. 7B, lane 4). We conclude from this experiment that SarA can support excisive recombination in the absence of Xis.

Discussion

Several observations suggest that SarA may function as an architectural protein rather than a classic transcription factor. For example, the molecular weight of SarA is similar to several *E. coli* histone-like proteins, and to the extent that the SarA binding site has been defined, putative binding sites are widely disbursed throughout the *S. aureus* genome both within and between open-reading frames. There is also evidence that functional SarA binding may involve distinct topological features in the target DNA (Fujimoto *et al.*, 2000). It was also recently demonstrated that the post-transcriptional persistence of

mRNA is *sarA*-dependent (Roberts *et al*., 2006). All of this is consistent with the observation that SarA contributes to the regulated expression of >100 genes that include not only genes encoding recognized virulence factors but also genes involved in essentially all other cellular processes (Dunman *et al*., 2001).

The studies described here were intended to address two issues, the first being to determine the amount of SarA present in a single *S. aureus* cell and whether the DNA binding activity of SarA varies in a growth phase-dependent manner. Based on a competitive ELISA employing a SarA-specific antibody, we estimate the number of SarA molecules in a single cell at ~50 000, which is significantly higher than a typical transcription factor but similar to accessory DNA binding proteins in *E. coli*. For example, the *E. coli* HU and Fis proteins occur in ~30 000 copies per cell (Johnson *et al*., 2005). Both HU and Fis are also DNA binding proteins that influence gene transcription in *E. coli* in a manner similar to SarA in *S. aureus* (Dorman and Deighan, 2003). We also found that neither the intracellular concentration nor the DNA binding activity of SarA changes significantly in a growth phase-dependent manner at least under standard *in vitro* growth conditions.

The experiments we report focused on the commonly used 8325-4 laboratory strain RN6390, and it is clear that this strain has characteristics that distinguish it from clinical isolates of *S. aureus*. These include mutations in *rsbU* and *tcaR*, both of which contribute to regulatory circuits in *S. aureus* (Oscarsson *et al*., 2006). This leaves open the possibility that the results we report are not representative of clinical isolates. However, in previous experiments comparing lysates prepared from RN6390 with those prepared with the clinical isolate UAMS-1, we were unable to demonstrate a significant difference in either the overall amount or the growth phase-dependent production of SarA (Blevins *et al*., 1999). This is consistent with subsequent reports indicating that strain-dependent differences between clinical isolates like UAMS-1 and RN6390 are due to differences in the production of other regulatory proteins (e.g. SarS) rather than differences in the production of SarA itself (Oscarsson *et al*., 2006; Gustafsson *et al*., 2009).

While we did not observe significant differences in the SarA binding activity during different growth phases, we did demonstrate that SarA binding is responsive to both oxidative stress and pH. This was demonstrated by EMSA using each of three known SarA targets (*agr* B1/B2, *cna* and *sspA*). In general, increasing the concentration of reducing agents or decreasing pH increased SarA's binding affinity to DNA. In both these experiments and those discussed above, whole-cell lysates were used in our EMSA experiments. This leaves open the possibility that alterations in SarA binding were associated with alterations in the interaction between SarA and some as yet unidentified cofactor. However, the fact that no binding was observed with extracts from the UAMS-957 *sarA* mutant in any of these experiments demonstrates that SarA itself is necessary if not sufficient for binding. The fact that mutation of the cysteine at position 9 of SarA abolished these effects provides further support for this hypothesis. This is similar to redox signalling through intramolecular disulphide bond formation of the transcription factor OxyR, which is responsive to H_2O_2 (Zheng *et al*., 1998). SarA functions as a dimer, and the cysteine at position 9 is located near the dimerization region (Liu *et al*., 2006). This suggests that changes in redox potential may alter the DNA binding activity of SarA by altering its ability to form a functional dimer. If disulphide bond formation is involved it would have to be intermolecular. Another mechanism for the changes in binding affinity would be via cysteine sulphenic acid. These moieties have been shown to affect the DNA binding of several eukaryotic transcription factors (Claiborne *et al*., 1999).

Although the production of SarA and its DNA binding activity were constant under standard *in vitro* growth conditions, the fact that binding was responsive to changes in redox potential is important in that *S. aureus* encounters varying oxidative states while growing *in vivo* depending on the stage and type of infection (e.g. during the adaptive response to growth in blood versus a biofilm versus inside an abscess versus inside a host cell). In fact, one important issue with respect to *S. aureus* regulatory circuits is the need to evaluate current regulatory paradigms defined by *in vitro* experiments in the more relevant context of growth *in vivo*.

Using EMSA conditions from Rechtin *et al.* (1999), SarA was found to bind to *attL* with an estimated apparent $K_D = 0.1$ nM and without the formation of complexes that resemble the Int-IHF–*attL* DNA intasome (data not shown). However, SarA–*attL* complexes resembling intasomes were formed in the presence of 5 mM spermidine and salmon sperm DNA. We surmise that spermidine binds DNA, reduces non-specific binding by SarA and stabilizes SarA–*attL* complex formation. The salmon sperm DNA present in the reaction may also compete for SarA and perhaps select for the most stable SarA–*attL* complexes, which would explain the high concentration of SarA needed to produce a shifted *attL* band. Although the exact stoichiometry of SarA : DNA in the *attL* complex is unknown, it is reasonable to assume that multiple SarA dimers can occupy the 179 bp *attL* DNA fragment. We also demonstrate that SarA can replace the activity of Xis in the *in vitro* intramolecular recombination reaction. To understand how SarA may stimulate excision recombination, we noted that two Xis monomers can bind to specific sites within *attR*, designated X, and bend DNA up to 140° (Thompson and Landy, 1988). The sequence within X is

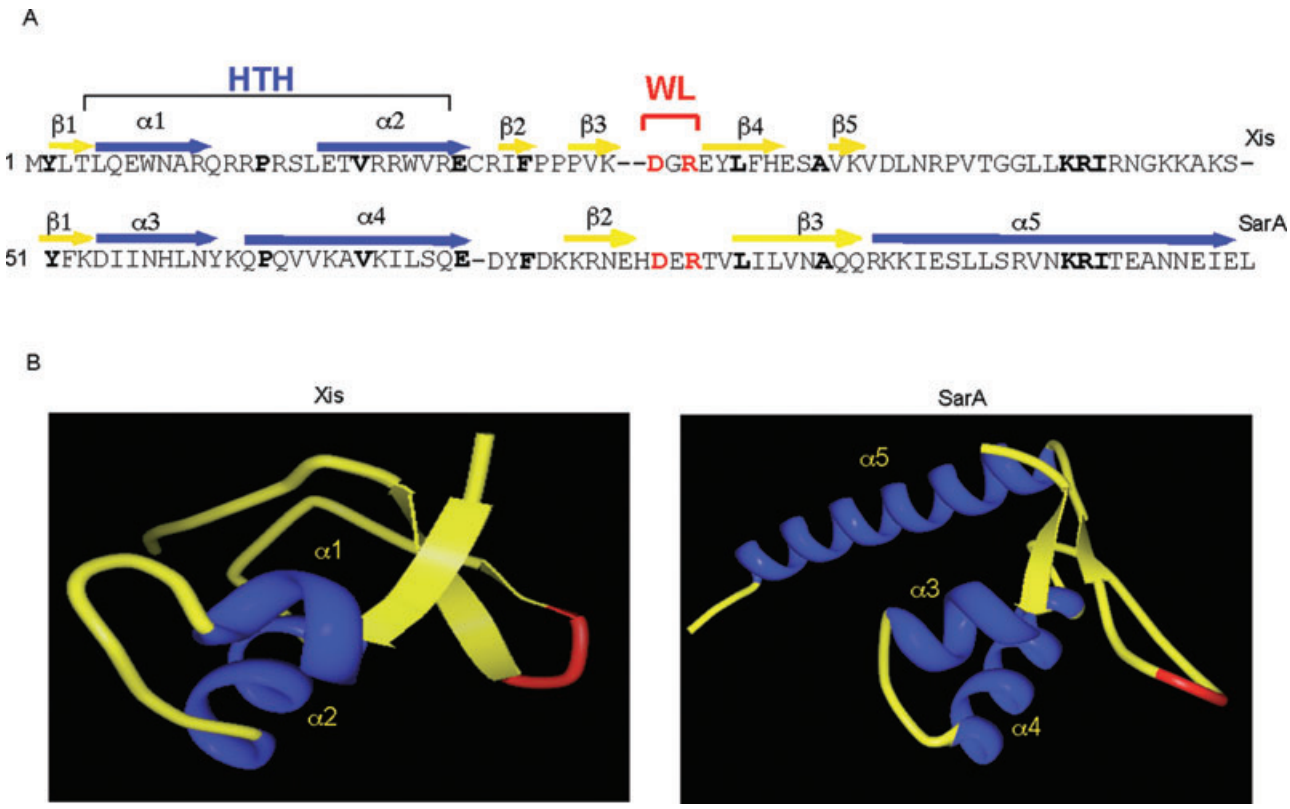


Fig. 8. A. Sequence alignment of the C-terminal end of SarA and Xis generated using the program ClustalW (http://www.ebi.ac.uk/slustalw/index.html). Identical residues are bold highlighted and secondary structural motifs are shown above each sequence as presented in Liu *et al.* (2006) and Sam *et al.* (2004). HTH, helix–turn–helix (blue); WL, winged loop (red).
B. Protein models of C-terminal domain generated by protein workshop (http://www.rcsb.org/pdb). Right, Xis, helices (blue), beta sheets (yellow arrows), conserved winged loop region (red).

AT-rich (25% GC, 75% AT) similar to *S. aureus* genomic DNA. There is an A-tract within X similar to the binding site identified by exponential enrichment experiments (Sterba *et al.*, 2003), which may be a preferred binding site for SarA. Based on this, we propose that SarA may bind and bend DNA within the Xis binding site thus stimulating Int recombination.

Liu *et al.* (2006) compared the SarA and SarR crystal structures, classifying these proteins within the same winged helix family. SarA and SarR share conserved 'DER' residues within the wing (β-hairpin) loop (Cheung *et al.*, 2004; Liu *et al.*, 2006). R90 of this triad is a functionally essential residue for SarA's DNA binding activity (Liu *et al.*, 2006). Superposition of the SarR dimer with CAP-DNA complex led to the prediction that the conserved DER residues on the wing loop, D86, E87 and R88 may interact with the minor groove of DNA (Liu *et al.*, 2001). Xis and SarA share a winged-helix DNA binding motif and both proteins possess loops protruding from the wing (Sam *et al.*, 2004; Liu *et al.*, 2006). Primary amino acid sequence alignment using ClustalW of the C-terminal 74 amino acids of SarA and full sequence of Xis reveal that the two proteins align not only by sequence but also based on secondary structure (Fig. 8A). This includes both the DNA recognition helices of the helix–turn–helix and the wing loop. Within the Xis wing loop, two of the three conserved 'DER' residues are present, D37 and R39. R39 interacts with the minor groove of DNA through direct base- and water-mediated contacts and does not contribute to specific contacts of Xis to DNA (Sam *et al.*, 2004). Additionally, similarities between SarA and Xis can be seen in a representation of their crystal structures (Fig. 8B). Orienting α-helix 4 of the C-terminal end of SarA and α-helix 2 of Xis at a similar angle show that the helix–turn–helix motif for both proteins are positioned away from their respective winged loops. This common feature may explain the overlapping function of SarA and Xis.

Our results support the hypothesis that SarA functions as an accessory protein, although we do not have an explanation for SarA's evolutionary relationship to Xis, as this protein is considered an RDF encoded by bacteriophage lambda similar to the majority of other RDFs that are encoded and produced by bacterial viruses. We believe that a potential functional role of SarA is architectural and may extend to biological processes including protection

against DNA damage by reactive oxygen species and, in response to the physiological state of the cell (e.g. redox and pH), controlling the structure of the *S. aureus* chromosome to mediate bacteriophage strand exchange events. Thus, our results illustrate a possible link between SarA DNA binding activities, reactive oxygen-induced DNA damage and the stimulation of DNA repair enzymes by derepression within *S. aureus*.

Experimental procedures

Bacterial strains and culture conditions

The *S. aureus* strains RN6390 and its isogenic *sar* mutant (UAMS-957) (Blevins *et al.*, 2002) were grown in 100 ml tryptic soy broth at 37°C with vigorous shaking (200 r.p.m.) in 500 ml baffled flasks. Culture growth was assessed by light scattering at 595 nm. Recombinant wild type and C9A SarA proteins were expressed from plasmid pET-DB in BL21 (DE3) pLysS *E. coli* cells and purified as described in Rechtin *et al.* (1999).

Western blot analysis

Anti-SarA IgG production was described in Blevins *et al.* (1999). Cell-free lysates of *S. aureus* strains RN6390 and UAMS-957 were prepared as described in Morfeldt *et al.* (1996), as well as in Blevins *et al.* (1999). Proteins were resolved by 12% tricine-SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to Immun-blot PVDF membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 5% w/v dry milk in TBST (10 mM Tris-Cl, pH 7.5, 0.9 mM NaCl, 0.05% Tween-20), washed with TBST and incubated with a 1:4000 dilution of anti-SarA IgG in TBST. Human Fc fragment was added to block binding to protein A. Goat anti-rabbit horseradish peroxidase (Bio-Rad) was used as the secondary antibody. Blots were developed with the ECL kit (Amersham Biosciences) according to the manufacturer's instructions and detected with X-ray film.

Competitive ELISA analysis

Pure SarA of 20 ng was bound to Immulon two 96-well plates (Dynex Technologies, Chantilly, VA) in 20 mM sodium carbonate, pH 10.0 overnight. The wells were washed with three times with TBST and blocked for 3 h with TBST-B (TBST containing 0.25% BSA). *S. aureus* strains UAMS-179 (RN6390, *spa*) and UAMS-284 (RN6390, *spa, sar*) were grown in tryptic soy broth. At optical densities of 0.1 (pre-log) and 1.5 (late-log) cells were harvested by centrifugation and the pellets frozen at –20°C. The remainder of the culture was allowed to grow overnight (stationary) and cells were harvested. For each time point an optical density equivalent of 3 was harvested (Blevins *et al.*, 1999). An aliquot of cells was diluted serially and plated on tryptic soy agar and counted to determine the number of cells used for the lysates. Cells were lysed with 0.5 ml of 25 mM Tris-Cl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 5 mM DTT and 1 mM PMSF in a Fast-Prep FP120 (QBiogene) in Fast-Prep Blue tubes at 6 m s^{-1} for 40 s. Lysates were cooled on ice, then insoluble material removed by centrifugation. Anti-SarA IgG (final dilution of 1:50 000) was incubated with the UAMS-284 lysate (negative control), the UAMS-284 lysate containing purified SarA (positive control) and the UAMS-179 lysate (experimental) on ice for 2–3 h prior to adding to the ELISA plate for another 2–3 h. The wells were washed three times with TBST-B. Goat anti-rabbit horseradish peroxidase secondary antibody (1:1000 dilution) in TBST-B was incubated for 30 min, followed by three washes with TBST and three washes with TBS. 100 µl/well of a solution consisting of 10 mg of SIGMAFAST o-Phenylenediamine dihydrochloride in 25 ml of 100 mM citrate, pH 5.5, plus 20 µl of H_2O_2 was used to develop the plate. The horseradish peroxidase reaction was quenched with 50 µl of 2 N HCl. Optical densities were determined at 490 nm in a Tecan UltraEvolution plate scanner.

Electrophorectic mobility shift assays

Three *cis*-acting elements shown to be bound with high affinity by SarA were used (Sterba *et al.*, 2003). The sequences of them are shown: *agr* B1/B2 ATGTTAAAATATTAAATACAAA TTACATTTAACAGTTAAGTATTTATTTCCTACAGTTA; *cna* TGTATATATTTTGCATAATAAAATAATAATATGAATTTTTGAT AAATTTCATTGAAT; *sspA* AAAAATTTTTATTGTTATATTTA ACTTGTAAATAAATTTTTTGGAGGTTTTTAGATGA.

The EMSAs were performed as described in Rechtin *et al.* (1999). Briefly, the oligonucleotides were synthesized, end-labelled with ^{32}P and purified following denaturing polyacrylamide gel electrophoresis. Complimentary DNAs were annealed, purified by native gel electrophoresis and utilized in the binding experiments. Limiting concentrations of DNA (< 10 pM) were used. Various concentrations of SarA were incubated in a 20 µl reaction with ^{32}P-labelled DNA and in buffer containing 10 mM HEPES, pH 7.6; 1 mM EDTA; 2 mM DTT; 50 mM KCl; 0.05% Triton-X-100; 5% glycerol. For the EMSAs utilizing cell-free lysates, 1 µg of poly dI/dC was added to bind non-specific DNA binding proteins. For the redox experiments, the 2 mM DTT was eliminated and the varying redox reagents were added at the concentrations described in the figures. For the pH experiments, different buffers were substituted for HEPES. The following buffers were used for the different pH conditions: pH 5.5 – citrate-phosphate, 6.0 – maleate, 6.5 phosphate, 7.0 – phosphate, 7.5 – Tris-Cl, 8.0 – Tris-Cl, 8.5 – boric acid-borax, 9.0 – ammediol, 9.5 – borax-NaOH, 10.0 – glycine-NaOH. Buffers were prepared according to Gomori (1955). Binding reactions were allowed to equilibrate for 30 min prior to electrophoresis. Bound products were separated from free DNA on a 6% native polyacrylamide (50:1 acrylamide : bisacrylamide) in 0.5× tris-borate-EDTA. Gels were run at 200 V and temperature maintained at 16°C with a circulating water bath. Resolved gels were dried and the products visualized by phosphorimaging. The amount of bound DNA was calculated from the reduction in the unbound DNA and plotted against the respective SarA concentrations. Stoichiometric determinations were done as described above.

Lambda Integrase, IHF and SarA attL *EMSAs*

attL DNA substrates of 179 bp were double end-labelled by enzymatic transfer of radiolabelled phosphate using [γ-^{32}P]-

ATP and T4 DNA ligase as recommended by manufacturer (NEB). 10 µl reactions contained: 50 mM Tris-Cl (pH 8.0), 11 mM boric acid, 5 mM spermidine, 60 mM KCl, 0.1 mg ml^{-1} BSA, 13.5% glycerol and 1 nM *attL* DNA. When appropriate, 35 nM Int and 40 nM IHF were added to reactions. The amount of SarA is shown above each gel. Reactions were first assembled as a DNA buffer premix followed by the addition of non-catalytic DNA binding proteins and initiated by the addition of Int or corresponding dilution buffer. Reactions were incubated for 30 min at 37°C and protein–DNA complexes were separated on 5% (29:1) acrylamide : bisacrylamide gels with 0.5× TBE at 4°C, gels were dried and visualized by phosphorimaging.

Recombination/excision assays

The 20 µl reactions contained: 0.6 µg pLR110, 100 nM lambda integrase, 17.5 nM IHF, 50 nM Xis (where applicable) and SarA added to final concentrations as shown above each gel image. Reaction conditions are as follows: 115 mM KCl, 5 mM spermidine, 0.43 mg ml^{-1} BSA, 7.5% glycerol, 55.5 mM Tris-Cl (pH 8.0), 44.4 mM boric acid and 1 mM EDTA. Reactions were initiated by the addition of Int, and incubated at 37°C for 1.5 h. Recombination reactions were extracted using phenol-chloroform, ethanol precipitated, and digested with EcoRI previous to separation by 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

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